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A new diagnostic tool for rapid and accurate detection of *Mycobacterium tuberculosis*

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Abstract   *Mycobacterium tuberculosis*, acid fast bacilli from the family of *Mycobacteriaceae*, is the causative agent of most cases of tuberculosis. Tuberculosis, as a communicable disease, remains a serious public health threat, killing more than one million people globally every year. Primary diagnosis of tuberculosis bacilli (TB) relies mainly on microscopic detection of acid fast bacilli (AFB), but the method suffers from low sensitivity and the results largely depend on the technician's skill. New diagnostic tools are necessary to be introduced for rapid and accurate detection of the bacilli in sputum samples. We, in collaboration with Anda Biologicals, have developed a new platform, named as "Patho-tb", for rapid detection of AFB with high sensitivity and with low dependence on human skills. Evaluation of Patho-tb test performance was done in two settings: (1) primary field study conducted using 38 sputa from high TB prevalence area of Iran (Zabol city near to the Afghanistan border), and (2) main study conducted using 476 sputa from Tehran, capital of Iran. Patho-tb was applied for processed sputum samples in parallel with routine diagnostic methods (including AFB microscopy, culture and PCR). All test results were compared to final clinical diagnostic state of an individual and diagnostic sensitivity (DSe), specificity, positive predictive value, negative predictive value and accuracy of each test results were calculated using standard formulations. Analytical sensitivity and specificity of the Patho-tb test were also determined. Calculated values for five above mentioned parameters are as follows: for field study: AFB (DSe: 29.6, DSp: 81.8, PPV: 80, NPV: 23.1, AC: 44.7), Patho-tb (DSe: 63, DSp: 72.7, PPV: 85, NPV: 44.4, AC: 65.8), and for main study: AFB (DSe: 86.1, DSp: 99.4, PPV: 98.5, NPV: 93.9, AC: 95.2), Patho-tb (DSe: 97.4, DSp: 92.9, PPV: 86.5, NPV: 98.7, AC: 94.3). Reproducibility of Patho-tb test results were near to 100% (Cohen's kappa value between 0.85 and 1). The detection limit of Patho-tb test with 100% positivity rate was 3×10^3 cells/ml of sputum. In the field study,

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Patho-tb test was 33.4% more sensitive than AFB microscopy, while the improvement was only 11.3% during the main study. Patho-tb results are easy to interpret and the test can be merged with other screening tests, like AFB. Totally, Patho-tb test alone or in conjunction with AFB microscopy is a useful screening tool for TB detection especially in poor geographical lab conditions.

1. Introduction

Traditionally, the detection of *Mycobacterium tuberculosis* bacilli, MTB, in sputum establishes the diagnosis of TB. This detection is made by the microscopic examination of sputa and the visualization of acid fast bacilli (AFB) followed by culture to confirm the diagnosis (Schluger, 2008; WHO, 1998), although it is now recognized that up to 22% of total TB cases may be culture negative (CDC, 2006). Due to the cost and difficulties of managing a reliable culture facility in many TB-endemic countries and because of the length of time required before obtaining results, all the prevailing institutions agreed that diagnosis must be based on AFB microscopy in rural areas (Odubanjo and Dada-Adegbola, 2011; Shea et al., 2009). This method nevertheless is tedious, tiring, time consuming and lacks sensitivity: globally only 45.6% of all new and relapsed TB cases in 2004 were smear-positive (WHO, 2006). Inexpensive rapid tests based on the detection of anti-TB antibodies in the patients' blood have been developed, but most performance studies reported much lower sensitivity and specificity values than for AFB microscopy. Some rapid tests seem to have good sensitivity but lack acceptable specificity to be useful in most conditions (Adjei et al., 2003). Other more sensitive and specific diagnostic tools, such as direct PCR amplification tests (American, 1997), were developed with a sensitivity higher than 90% and a specificity close to 100% in smear positive cases. These tests are ideal for differentiating true TB infection from infections with non-tuberculosis bacilli, NTB, but their overall sensitivity remains too low. Lower sensitivity in smear negative cases, higher cost, time required to perform and technical or material needs, in collection, make them poor candidates for replacing microscopy. We developed a test for the direct detection of bacilli in sputum that is as sensitive as the Ziehl–Neelsen method, but devoid of a strong dependence on technicians' skills and material. The new Patho-tb test was developed in collaboration between our TB laboratory and Anda-Biologicals (France). Our choice was a simple method based on filtration of treated samples followed by visualization of the bacilli trapped on the filter by an immuno-chromatographic method using gold conjugate. In this paper, we report first some practical aspects of the Patho-tb test and then the results of a trial made to compare culture, AFB microscopy and the new test (see Fig. 1).

2. Materials and methods

2.1. Materials

"Mycobacteria Patho-tb" rapid diagnostic kit, positive and negative controls (in freeze-dried format with sodium azide), solutions for sample processing; neutralizing and dissolving, and other required reagents were provided by Pasteur Institute of Iran (PII) and Anda Biologicals, (www.anda-biologicals.com), as a compact ready to use kit. Purified rabbit anti-TB antibodies were produced in PII and colloidal gold conjugated goat anti-rabbit antibodies were purchased from sigma (G7277 SIGMA). Ziehl Neelsen (ZN) acid-fast staining was performed according to the CDC manual (Kent and Kubica, 1985). AFB microscopy results were categorized as negative, equivocal, $1+$, $2+$ and $3+$. For statistical comparisons the results were transformed into binary format (negative or positive).

2.2. Culture conditions

Liquefied/decontaminated sputum samples were cultured on Lowenstein-Jensen slant media for isolation and identification of mycobacteria, according to the recommendations of both CDC (Kent and Kubica, 1985) and IUATLD (Rieder et al., 1998). The culture results were classified as negative when no grown colonies were seen on the slant after 8 weeks of incubation. Grown colonies were characterized and identified to species by colony characteristics on culture slant media and biochemical reactions. Contaminated culture results were excluded from analysis.

2.3. PCR assay

Repetitive insertion sequence, IS6110-based PCR detection of pathogenic TB was done (Abraham et al., 2012). DNA was extracted using "High pure PCR template preparation kit" (Roche; product No: 11796828001), and primer sets were purchased from "DNA technology, Moscow, Russia". Oligonucleotide primers for PCR amplification of 123 bp target fragment were as follows: forward primer: 5′-CCTGCGA GCGTAGGCGTCGG-3′ and reverse primer: 5′-CTCGTCC AGCGCCGCTTCGG-3′. For further details see studies of Bi et al. (1995) and Eisenach et al. (1990).

2.4. Patho-tb kit content

Patho-tb kit is made up of three main components: the filtration system, the decontamination system, and the rapid test system. The filtration system, (the kit platform), consists of a plastic cartridge; a funnel; and a glass fiber. The cartridge has a cubic empty space usually fills with filtering and absorbing layers and there is a circle opening located at the center position. The cartridge fills in with one layer of thick porous absorbing pad at the bottom and a layer of 0.45 micron pores nitrocellulose membrane on the top. The funnel application improves the sensitivity of the assay through focusing sample and other test solutions in a small circle area (about 0.3 cm in diameter). Enough glass fibers are also included in the kit that have application only in the first step of the assay and acts
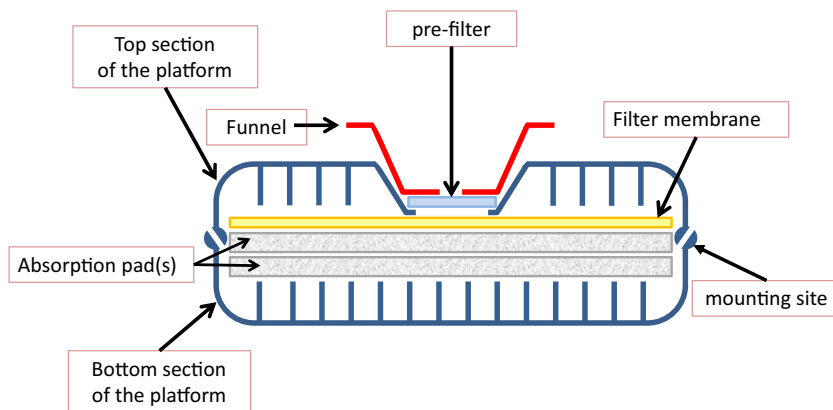
Figure 1 Patho-tb kit platform components. Vertical section from middle region of the platform is presented here. Body of the platform is composed of the two main parts: a bottom part and a top part. The two parts will mount to each other using mounting site. One or several absorbing pads are placed inside the platform and a nitrocellulose membrane filter exists that was located above the pads. In the top-middle position of the platform is a hole that was allocated for transferring sample and other reagents on the membrane. Pre-filter, made from glass fibers, was only devised for the first step of the assay, and its application is recommended for removing large particulate materials that may be present in sputum samples and can clog up the main filter membrane. An elastic funnel is also designed to focus sample and other reagents into a small circle area on the filter, and therefore amplify the observed color signal and also to economize in materials and reagents.

as a pre-filter to remove large particulate matters from processed sputum samples that may potentially clog the main membrane (filter). The decontamination system, (the reagents needed for sample preparation), includes the materials that are needed to prepare kubica solution. The method is used to digest and decontaminate viscose and highly contaminated sputum samples (Morcillo et al., 2008). Clinical specimens are usually associated with contaminating microorganisms and organic debris. Kubica solution is used for killing any contaminating, fast growing microorganisms present in the samples, and for liquefying organic debris that may surround the mycobacteria. Centrifugation of liquefied sample further concentrates the bacilli in the pellet and improves the possibility of obtaining positive results through the later recognition/detection steps. The rapid test system, (the main reagents to carry out the Patho-tb assay), consists of dissolving and washing solutions; negative and positive controls; colloidal gold conjugated goat anti-rabbit antibodies; and a purified antibody against MTB proteins (antigens) obtained via affinity chromatography.

2.5. Antigen preparation

Mycobacterial cells, grown in 7H9 broth culture (after four weeks of incubation at 37 °C), were harvested and washed 3 times using phosphate buffered saline (PBS), (pH: 7.3), centrifuged (3000 rpm, 15 min), and the pellet was mixed with purification buffer (NaN3: 0.002 g/dl, Triton X100: 1%, Glycerol: 10%, DTT: 10 mM, PMSF: 1 mM and EDTA: 20 mM, pH: 7.4), freezed (5 min, liquid nitrogen) and thawed (5 min, boiling water) 30 cycles. The suspension was then sonicated using a probe sonicator (50 Hz for one hour, under safety cabinet), and centrifuged (4000 rpm, 15 min). The pellet was discarded and the supernatant was mixed with absolute ethanol, ETOH, (volumes of ETOH [99%, −20 °C] were added to the supernatant until the final concentration of 60% ETOH was achieved), and centrifuged (15000 rpm,

30 min). The protein rich pellet was resuspended in PBS buffer (pH: 7.3), and dialyzed in three steps against the buffer. Protein concentration of the final solution was determined using Bradford assay.

2.6. Preparation of TB-specific antibodies

Antibodies were used for recognizing and binding to the mycobacteria cells retained on nitrocellulose membrane pores. Anti-TB antibodies were produced in PII TB reference laboratory. Polyclonal TB-specific antibodies were raised in rabbits using a well-established immunization protocol. Briefly, 250 µl from the working solution of purified mycobacterial proteins (1 mg/1 ml) was emulsified in 250 µl of incomplete freund's adjuvant and subcutaneously injected to each rabbit four times with three week intervals (at 0, 21, 42 and 63 days). TB-specific antibody levels in immunized rabbit serums were continuously monitored using ELISA system. Finally, blood from rabbits was obtained at 66 days, and the serum fractions were separated using centrifugal force (2500 rpm, 20 min).

2.7. Purification of the antibodies

Rabbit sera were subjected to further purification using CNBr-activated TB-antigens coupled Sepharose 4B column chromatography. The precipitated dialyzed protein solution (TB-antigens) was coupled to CNBr-activated Sepharose 4B beads (2.4 mg protein per 1 g beads) and the remaining free sites were blocked using ethanolamine (3.4 ml ethanolamine added to 996.6 ml carbonate–bicarbonate buffer, 0.05 M). The column was washed with glycine buffer (0.2 M, pH: 2.8) until the optical density (OD) of eluted solution read 0 value at 280 nm indicating complete removal of loose bound proteins from the column. The column was washed with tris buffer (0.1 M, pH: 7.3) and filled with 500 µl of rabbit immune sera, incubated at RT for 1 h. The column washed with tris buffer to remove unbound proteins. To elute bound Ab(s) glycine buffer

(0.1 M, pH: 2.8) was added and eluted solution was monitored using spectrophotometer until the OD reached to zero value. Tubes containing TB-specific polyclonal antibodies were collected and dialyzed against PBS (membrane pore size 10,000, overnight incubation, repeated three steps). ELISA assay was used for Ab titration and the Abs were absorbed for removing non-specific Abs.

2.8. Absorption process

For achieving the highest specificity of the TB recognition and to eliminate the non-specific reactions, the purified polyclonal antibodies were absorbed against TB-negative (smear-, culture-, PCR-), diluted, and pH adjusted (pH: 9.6), sputum samples (300 sputa from our sample bank that had been double-checked and confirmed as smear, PCR and culture negative samples). In other words, a TB-negative sputum refers to a sputum sample collected from a TB-negative individual, processed through a standard procedure and evaluated using three tests and retained negative results by all three methods, AFB, culture and PCR analysis. Several ELISA plates coated with the sputa were used for absorption process. Briefly, initially processed, then pooled, TB-negative sputa were diluted using carbonate/bicarbonate coating buffer (Na_2CO_3: 3.03 g; $NaHCO_3$: 6.0 g; pH: 9.6; final volume 1000 ml), transferred to the wells of microtiter plate (120 μl/well) and incubated for 24 h at 4 °C. Unbound sites were blocked using bovine serum albumin (Bovine serum albumin fraction V: 1% and 200 μl/well; o/n; at 4 °C). Excess liquid discarded from the wells (under safety cabinet), Ab solution was added and incubated (100 μl/well; 2 h; at RT, with gentle agitation). The absorption process was repeated two times in another set of coated plates to remove existing non-specific Ab(s). Finally, the absorbed Ab was collected and aliquoted in 1 ml cryotubes and kept at −20 °C.

2.9. Population and samples

Evaluation of Patho-tb kit performance was carried out in two separate times and settings. Primary study for evaluation of Patho-tb diagnostic performance was conducted on April 2011 using sputum samples collected from 38 TB-suspected individuals from Zabol city (located near to the Afghanistan border of Iran), with high TB prevalence (109.7 per 100,000 population) (Iran's Ministry of Health Report, 2011; Metanat et al., 2012). Three consecutive samples were collected on three separate days. Under the geographical conditions the only diagnostic measure could be acid fast staining applied to the partially processed sputum samples and usually observed by inexperienced staffs (compared to reference laboratories). Patho-tb test was applied in parallel with routine acid fast bacilli detection task. However, PCR-based detection of TB bacilli for each sputum sample was done in reference laboratory in Pasteur Institute of Iran (in Tehran capital of Iran). Main study was started from Jan 2011 and lasted up to the end of 2014. Totally 476 fresh sputa samples were collected from active pulmonary tuberculosis (PTB) suspected patients, from Tehran province, referred to TB reference laboratory by referring laboratories or clinicians, examined for AFM, TB-specific culture, PCR amplification and also by Patho-tb assays. Repeated sample collection was only done if the primary sample results were negative or unreliable. The definitive diagnosis of active TB-infection, is based on the collective results of the laboratory tests, CXR, and patient's clinical manifestation.

2.10. Sample processing

Sample collection and processing were done according to WHO, CDC recommendations. A code number was assigned to each sputum sample and assayed in a blinded manner by operators and the results were decoded during final analysis. Kubica solution was used for sample digestion and decontamination according to the kit manual. Briefly, equal volumes (2 ml) of sputum and freshly prepared kubica solution were mixed in a 50 ml conical centrifuge tube and incubated at room temperature for 20 min. The mixture was neutralized with 18 ml of neutralizing buffer and centrifuged for 20 min at 4000 rpm. The supernatant discarded and the resulting pellet was resuspended in 1–5 ml of neutralizing buffer (depending on the pellet viscosity). Equal volumes (0.1 ml) of the resuspended pellet and dissolving solution were mixed in a boiling-resistant microtube and floated in boiling water bath for 20 min using a floating support. The remaining pellet was used for microbiological examination and acid-fast microscopy.

2.11. Patho-tb assay procedure

The cartridge was mounted using one piece of pre-filter and a funnel. Processed and boiled sample (100 μl) was immediately loaded on the pre-filter surface and allowed to be absorbed. After washing (3 times using 50 μl wash buffer), pre-filter was removed and the funnel was mounted again. Anti-TB antibodies (50 μl) were then added and allowed to drain. Two drops of anti-rabbit Ab-gold conjugates were added followed by a washing step. The funnel was removed and washed again. Development of a red-pink color on the center of membrane indicates the presence of tuberculosis bacilli in the sample. Wash buffer is composed of PBS supplemented with 0.1% Tween 20 and sodium mercurothiolate as preservative. Both, positive control (killed *M. tuberculosis* 1137 Ra) and negative control were included in the kit and processed according to the kit manual. Briefly, digestion/decontamination step was omitted and equal volumes (100 μl) of the two controls and dissolving solution were mixed, boiled and assayed in parallel with test samples.

2.12. Interpretation of the results

Developed central red-pink color was observed and judged in comparison with negative control result(s). Any red-pink ring around the test area was considered as an artifact. Doubtful results (weak red-pink signal) were re-assayed using new samples. The color intensity is proportional to the density of mycobacteria trapped in the filter. The results were expressed as negative or positive.

2.13. Repeatability of the assay

Repeatability defines as intra-laboratory variability of the results. Result variability may be divided into run-to-run variability with the same operator, inter-operator variability,

Table 1 Classification of cases using combination of test results (primary study).

Total cases	Diagnosis	PCR	AFB	Patho-tb	No. of cases
38	27 +	+	+	+	8
		+	FN	+	9
		+	FN	FN	10
	11 −	FP	FP	FP	2
		FP	−	FP	1
		FP	−	−	1
		−	−	−	7

FN: false negative; FP: false positive; +: true positive; −: true negative.

inter-equipments or inter-batch variability. Reference samples with long term stability under preservation conditions for measuring repeatability did not exist. Home-made reference samples using cultured mycobacteria diluted in confirmed TB-negative sputa showed low stability under preservation conditions. Notable signal reduction of the prepared reference samples was observed after three days of preservation at 4 °C or after 10 days preservation at −20 °C. Despite the difficulties, positive reference samples were prepared and aliquoted, using cultured *M. tuberculosis*, at final concentrations of 3×10^8, 1×10^7 and 1×10^5 cells/ml, diluted in TB-negative pooled sputa, preserved in −20 °C, and assayed utmost 10 days by different operators. Aliquots from the pooled sputa lacking MTB were used as negative control. The reference samples were assayed in parallel with the tests. Intra-assay or inter-assay agreement of the results from repeated measures was calculated using Cohen's kappa statistical test.

2.14. Analytical sensitivity and specificity

A homogenous suspension of *M. tuberculosis*, equal to one McFarland standard turbidity (3×10^8 cells/ml, in PBS), was prepared, from which, 10-fold serial dilutions were made and 100 μl from each dilution was mixed with 900 μl pooled, homogenized, TB-negative sputa, and assayed as field samples, in a blind manner, by different operators. Dilution factors were considered in the calculations of MTB density in reference sputum samples.

Cross reactivity of the test: In a comparative study, suspensions equal to one McFarland standard turbidity from the following species were prepared: *M. tuberculosis*, *Mycobacterium avium*, *Mycobacterium bovis*, *Mycobacterium bovis BCG*, *Mycobacterium fortuitum*, *Mycobacterium intracellulare*, *Mycobacterium kansasii*, *Mycobacterium scrofulaceum* and *Mycobacterium smegmatis*. Three dilutions (3×10^7, 3×10^5 and 3×10^3 cells/ml in PBS) from each species were prepared and assayed with Patho-tb and compared.

2.15. Diagnostic sensitivity and specificity

Culture is considered as a gold standard among laboratory tests for MTB, however, determination of condition index (presented as "diagnosis" in tables), for an individual relies on the collection of clinical manifestation and paraclinical test results, including AFM, culture, PCR, CXR, TST. Therefore,

true positive (TP), true negative (TN), false positive (FP) and false negative (FN) nature of the test results were determined through comparing each result with collectively determined infectious index of the patient.

3. Results

3.1. Primary study results

Result distribution of three MTB diagnostic tests, AFB; PCR; and Patho-tb, for 38 PTB suspected patients from Zabol is presented in Table 1. Performance characteristics of the tests are provided in Table 3.

3.2. Main study results

The main study was conducted in reference laboratory using fresh sputum samples from 476 PTB suspected patients. Result distribution of culture; AFM; PCR; Patho-tb tests for the patients is presented in Table 2. The data can be used for regeneration of full lists of results as a worksheet. Performance characteristic of the test is provided in Table 3.

3.3. Comparison of assay performances

Performance characteristics of the 4 tests in both primary and main studies are presented in Table 3.

3.4. Repeatability

TB-negative sputa were spiked and used as positive reference samples. Final concentrations of 1×10^8, 1.5×10^6 and 1×10^5 bacterial cells/ml were used to define intra-laboratory reproducibility of the Patho-tb assay. TB-negative sputa (smear-, culture-, PCR-), were also used as a negative reference sample. Results of positive and negative reference samples were used for calculation of agreement coefficient by Cohen's kappa statistical test. The calculated Kappa values for reference samples containing 1×10^8, 1.5×10^6 cells/ml were always equal to one, but for lower concentration, 1×10^5 cells/ml, the calculated Kappa value ranged from 0.85 to 1. Cohen's kappa usually ranges from zero to one. Zero shows overall disagreement, while one means full agreement between repeated measurements.

3.5. Analytical sensitivity

Pooled TB-negative sputa were spiked with serial dilutions of MTB. The reference samples treated the same way as worked with field samples. The detection limit of Patho-tb assay with 100% positivity rate was 3×10^3 cells/ml of sputum. Mean positivity rate obtained from concentrations of 3×10^2 cells/ml (equal to 300 mycobacteria on filter paper) was 32%. Please note that, the final density of MTB in 1 ml processed sputum (not in PBS) was considered in final report.

3.6. Analytical specificity

Cross-reactivity of recognizing rabbit polyclonal TB-specific antibody to non-pathogenic mycobacteria was assessed. Final

Table 2 Result Distribution of 4 tests for 476 PTB suspected cases (main study).

Total cases	Diagnosis	PCR	Culture	AFB	Patho-tb	No. of cases
476	151 +	+	+	+	+	111
		+	+	+	FN	1
		+	+	FN	+	18
		+	+	FN	FN	3
		+	FN	+	+	18
	325 −	FP	−	−	+	13
		FP	−	−	−	15
		−	−	FP	FP	1
		−	−	FP	−	1
		−	−	−	FP	9
		−	−	−	−	286

Diagnosis, in fact, represents the final infectious state of the individuals (used as gold standard to calculate PPV, NPV, DSe, DSp and AC). 151 out of 476 patients were diagnosed as active PTB (Prevalence: 31.7%). FN: false negative; FP: false positive; +: true positive; −: true negative.

signal strength was 10 to 100 fold lower in non-pathogenic mycobacteria compared to pathogenic species. That means very high density of non-pathogenic mycobacteria is required to possess interfering effects on pathogenic TB recognition. Descending order of final signal strength caused by non-pathogenic species is as follows: *M. avium* > *M. bovis* > *M. bovis BCG* > *M. fortuitum* > *M. intracellulare* > *M. kansasii* > *M. scrofulaceum* > *M. smegmatis*.

4. Discussion

The best diagnostic tool for TB detection is generally accepted to be culture (Schluger, 2008; WHO, 1998). Indeed, most authors have reported a good sensitivity (90–100%) for this method in pulmonary cases (Lu et al., 1996) and it is generally accepted that it is almost 100% specific. Nevertheless, the total number of positive TB cases that are culture negative in the USA reached 22% in 2005 (CDC, 2006) and certain studies

report sensitivities of 50% or lower in special patient groups (Delacourt et al., 1993; Burman et al., 1997; Chiang et al., 1997; Swanimathan and Umadevi, 1999). While it remains the gold standard for TB detection, more than 8 weeks length of time is required to yield results, and poor financial or staff resources in TB-endemic area does not usually support implementing the technique in the location. Therefore, AFB detection by microscopy is advocated in its place.

AFB microscopy is cheap, except for the microscope, requires little material and gives results in a day. On the other hand, it is a tiring and cumbersome technique that relies heavily on technician's skill, quality of chemicals and human interpretation. Since 1975 individual studies (Boyd and Marr, 1975; Lipsky et al., 1984; Levy et al., 1989; Karstaedt et al., 1998) reported a great disparity in the sensitivity values, from 33% to 80%, depending on the group studied, incidence of TB in the area, sputum dissolving techniques, staining techniques and the number of samples tested per patient. The most recent global report (WHO, 2006) indicates that even among PTB cases the sensitivity of this method is only 40–58%. Although AFB microscopy is believed to be very specific, however, the differential identification of mycobacterium species by morphological parameters is almost impossible.

In our study, DSe and DSp of AFB microscopy in poor area lab and in reference lab were 29.6%, 81.8% and 86.1%, 99.4%, respectively. The low DSe value (29.6%) of the method in poor area lab, compared to reference lab (86.1%), is indicative of determinant influence of technician's skill and working conditions on accuracy of the test results. Simultaneously, the DSe value for Patho-tb test results under the poor area lab conditions was 63%, that means Patho-tb test is twofold more sensitive than AFB microscopy. The DSe of Patho-tb test was also higher in reference lab conditions compared to AFB (97.4% against 86.1%). Negative predictive value of Patho-tb test is also higher in poor lab conditions compared to AFB microscopy (44.4% against 32.1%), and even in reference lab conditions (98.7% against 93.9%). Eighteen out of 21 AFB-negative samples (from reference lab) and nine out of 19 AFB-negative samples (from poor conditions) were accurately diagnosed as positive by Patho-tb test. In collection, Patho-tb test results are more sensitive and more accurate than AFB microscopy, but the intervals were obviously higher in

Table 3 Performance characteristics of culture; PCR; AFB and Patho-tb tests in diagnosis of active PTB.

	Method	Final interpretation (No. of specimen)				DSe (%)	DSp (%)	PPV (%)	NPV (%)	AC (%)
		TP	FP	TN	FN					
Primary study	AFB	8	2	9	19	29.6	81.8	80.0	32.1	44.7
	PCR	27	4	7	0	100.0	63.7	87.1	100.0	89.5
	Patho-tb	17	3	8	10	63.0	72.7	85.0	44.4	65.8
Main study	Culture	133	0	325	18	88.1	100.0	100.0	94.8	96.2
	PCR	151	28	297	0	100.0	91.3	84.3	100.0	94.1
	AFB	130	2	323	21	86.1	99.4	98.5	93.9	95.2
	Patho-tb	147	23	302	4	97.4	92.9	86.5	98.7	94.3

DSe: diagnostic sensitivity; DSp: diagnostic specificity; PPV: positive predictive value; NPV: negative predictive value; PCR: routine PCR for 123 bp IS6110 fragment; AFB: acid fast bacilli microscopy; Patho-tb: A new kit from Anda Biologicals. Culture for *M. tuberculosis* isolation was not possible during the primary study because of poor resource. PCR test for primary study was done on preserved samples, later in my reference laboratory, in Tehran. Primary study: 27 out of the 38 suspected cases (prevalence: 71%), were indexed as having active PTB and 11 cases were classified as negative. Main study prevalence of PTB was 31.7% (151 +/476−).

poor lab conditions (DSe: 63% against 29.6% and AC: 65.8% against 44.7%). The higher sensitivity and the higher negative predictive value of the Patho-tb test make it the best screening test among the three, especially in the poor conditions.

Contrary to the AFB microscopy, the Patho-tb test is a straightforward and easy to perform method, allowing an average of 30 samples to be treated simultaneously. Twenty minute heating of processed samples on boiling water makes the sample non-infectious, and therefore, protects technicians from acquiring the disease. The results of the tests are easy to interpret and rely far less on individual interpretation. The agreement of Patho-tb results (kappa coefficient) between different operators and between separate days was about 0.85 to 1 that indicates technician's skill independency of the test results. Inclusion of the Kubica solutions in the kit has the advantage of increasing the robustness of the kit by limiting lab-to-lab variations in decontamination procedures. The test results are also highly reproducible. All five technicians involved with the test in our lab indicated a preference for a less cumbersome technique. Treating 30 samples or more at the same time in about 2 h was greatly appreciated. The needed centrifuge and water bath are standard equipments in most field laboratories in our country. When all financial parameters are taken into account, the test is inexpensive and its selling price should be close to that of microscopy.

The preliminary field trial in Zabol (Table 1) was made under more difficult conditions. The equipments had lesser quality and the technicians were less experienced. The reported accuracy of the Patho-tb test (65.8%) is thus far higher than that of microscopy (44.7%). Although the Zabol trial was limited in scope, it shows that the Patho-tb test, as a screening assay, is more suitable for use in field labs than microscopy.

The higher sensitivity of Patho-tb test partly depends on the quality of recognizing antibodies included in the kit, which have been produced and purified in our lab. High quality TB-specific polyclonal antibodies were raised in rabbits, a good antibody producer. Several absorption steps were implemented to remove cross-reactive antibodies and minimizing background signals. However, cross-reactivity of the antibody to other mycobacteria is inevitable. Indeed, in a try in our laboratory, absorption of the rabbit antisera using non-pathogenic mycobacteria resulted in significant reduction in the test signal, and therefore, leads to reduction in the test sensitivity, thus, absorption with non-pathogenic mycobacteria was omitted. Presence of non-pathogenic mycobacteria in pulmonary samples is a rare event, but during examination of extra-pulmonary samples positive results should be related to the observed clinical signs and to the nature of the specimen.

PCR-based detection of MTB in sputum samples from the two study settings was done in the reference lab. The DSe and DSp values for PCR-based detection of MTB in samples from preliminary and secondary studies were 100%, 63.7% and 100%, 91.3%, respectively. The calculated accuracy of PCR assay in the field lab setting was 89.5%, that is significantly higher than the accuracies of AFB (44.7%) and Patho-tb (65.8%) tests, however, facilities for implementing the test were not available in the endemic area. In contrast to some reports from India about absence of even a single copy of IS6110 in 1% of MTB isolates, all of 514 MTB isolates in the current study were positive for the presence of the gene sequence (Balamurugan et al., 2006). In one study by analysis of 49 sputum samples, the rate of smear and culture negativity was 75%, of which, 85% yielded positive results using IS6110 based PCR (Abraham et al., 2012). The other survey by Farzam et al. (2015), has reported 95.1% and 42.9% sensitivity values of the IS6110 PCR assay for culture isolates and direct specimens, respectively. Indeed, IS6110 PCR based assay was able to detect 10 copies of MTB in the specimens (Nghiem et al., 2015).

5. Conclusion

In terms of ease of use, duration, ease of interpretation, robustness, reproducibility and record-keeping the Patho-tb test we developed meets or even exceeds AFB microscopy and culture. It was demonstrated that the Patho-tb test was 33.9% more sensitive and 21.1% more accurate than AFB microscopy. The test was technician's skill independent and can easily be incorporated in the general processing of sputa samples for TB diagnosis and is especially useful as a screening test for TB detection, especially in poor facility and poor human resource conditions.

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